

Are Lignins Optically Active?

J. Ralph, J. Peng, F. Lu and R.D. Hatfield.

Introduction

Lignin is a complex polymer produced in the plant cell wall by radical coupling reactions. It provides no nutritive value to ruminants and further ties up potentially digestible polysaccharides. Other plant polymers such as polysaccharides and proteins are synthesized by the plant with careful attention to structure. As such, their monomeric components and the polymers themselves, are optically active — that is, they have chirality, or “handedness” (as our right and left hands differ by being mirror images). It has long been assumed that lignins are not optically active since they derive from monomers with no chirality and appear to be the result of chemical reactions that are independent of enzymatic control. To date, however, there does not appear to be any rigorous proof of this assertion. Recently, the accepted derivation of lignins from non-(enzymatically)-controlled reactions has been challenged, and it is relevant to ascertain unequivocally whether lignin is, or is not, optically active.

Lignans, dimers of monolignols, are also produced by many plants, and are often in optically active forms. For example, the lignan pinoresinol, and its derived matairesinol, were found only as their (-)-isomers in suspension-cultured *Pinus taeda* (Eberhardt et al. 1993). A recent isolation of a “dirigent” protein from *Forsythia* which facilitates coniferyl alcohol radical coupling to pinoresinol in a regio- and stereospecific manner has lead the discoverers to extrapolate their lignan observations to lignins (Davin et al. 1997), as is discussed in an accompanying article [see p. 33]. Although their new paradigm may not require the resultant lignin to be optically active, the finding of chiral lignin would significantly strengthen the idea.

If lignification were to be controlled in a structural and stereochemical sense by dirigent proteins, it would be possible to alter the properties of lignin, potentially to our advantage, by targeting these proteins using biogenetic engineering methods. The question of lignin optical activity therefore needs to be addressed. This

report describes a two-pronged approach to resolve the issue as unequivocally as possible.

Materials and Methods

Isolated lignins were dissolved in 10:3 acetonitrile:water, a particularly good solvent for UV studies. DFRC conditions were those described previously (Lu and Ralph 1997b; Lu and Ralph 1997a). Circular dichroism (CD) spectra were run on an AVIV model 62A-DS circular dichroism spectrometer (Lakewood, NJ) driven by an Apple Macintosh computer running IGOR-Pro 3 software (Wavemetrics, Lake Oswego, Oregon). The lamp current was set at 26 amps; temperature was 25 °C; all conditions were standard. For lignin dimers, the solvent was acetonitrile (HPLC grade, Baker); for isolated lignins, the solvent was 10:3 acetonitrile:water. Sample concentrations were adjusted so that optical densities measured by the dynode were just below the maximum of 600 V at maximal absorptions in the spectral range. Spectra were obtained using 10-s averaging in 1 nm steps. Spectra were baseline subtracted (using scans of the blank solvents). Raw non-smoothed spectra are shown in the figures.

Results and Discussion

Of the various methods for determining optical activity, circular dichroism (CD) was chosen for its sensitivity and because the association of CD phenomena only with UV-active absorptions allows optical activity in lignins to be determined even in the presence of contaminating (optically active) polysaccharides.

Lignin dimeric degradation products. Dimeric lignin fragments were isolated from pine wood following DFRC (derivatization followed by reductive cleavage). As seen in Fig. 1, the bonds originally formed in the radical coupling step remain unaltered from their native states following this degradation. Determination of chirality in these compounds therefore reflects chirality in the original lignin. This is particularly clear-cut with b-5 and b-b-compounds (where the crucial bonds do not cleave), but less so

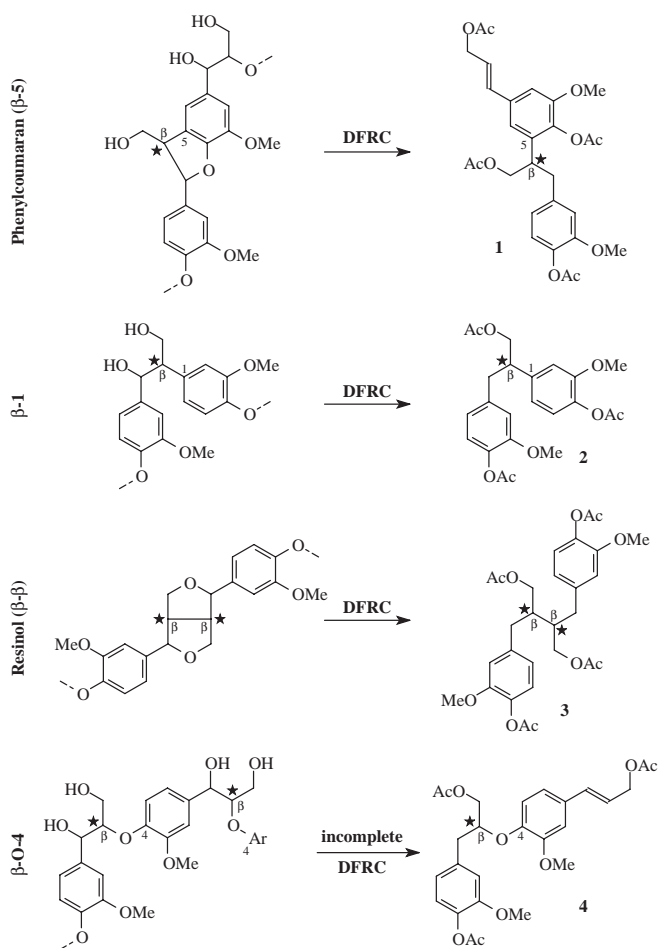


Figure 1. Dimeric DFRC products from various structural units in softwood lignins. Optical centers at β -carbons formed by radical coupling reactions during lignification are denoted with a ★. Note: the β -O-4-dimer **4** is only produced in small amounts from incomplete ether cleavage.

for β -1's (where there are still some uncertainties regarding how they arise) and, perhaps, β -O-4's (where it is less clear that the β -O-4 bond has not been broken and reformed in the production of the fragment dimers).

Figure 2 shows CD spectra of dimers **1-4** isolated from pine lignin following DFRC, along with spectra from both enantiomers of the β -5-dimers **1** (which were separated by chiral HPLC). The spectra are a little noisy, particularly near 200 nm, where the absorption is high and the total optical density of the sample limits the sample concentration that can be used for CD. However, high CD rotations are also expected at high absorption wavelengths, so high sensitivity is expected in this area. This may be seen from the 190-245 scans of the β -5-enantiomers **1** where the concentrations were reduced five-fold and

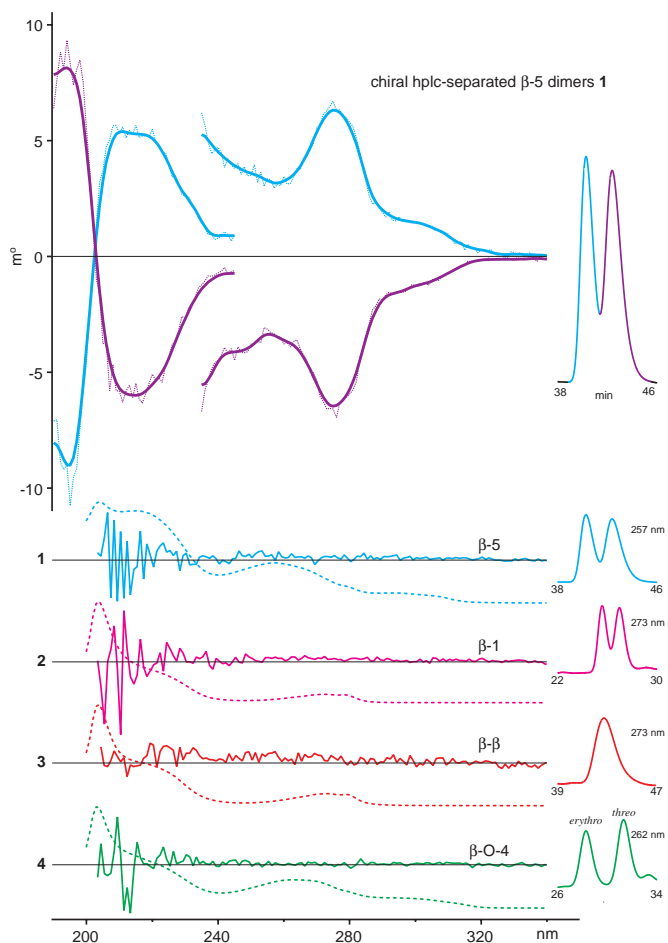


Figure 2. CD spectra of isolated DFRC dimers **1-4** along with the chiral-HPLC-separated β -5-dimer **1**. UV spectra are shown with dashed lines; the CD spectra are more noisy at high UV absorptions, but the CD rotations are also strongest in the region of strong UV absorption. Chiral-column HPLC traces are shown to the right of the figure; the β - β -enantiomers **3** were not resolved, nor were the β -O-4-enantiomers **4**, although the threo- and erythro- (syn- and anti-) diastereomers were. The resolved β -5-enantiomers **1** showed strong CD spectra; the region from 190-245 nm was run using one quarter the concentration used for the 240-350 nm region.

still produce pronounced CD rotations. At 280 nm, a maximum in lignin UV spectra, the noise level is sufficiently low to allow relatively sensitive determination of optical activity. No hint of such activity was found.

Of the linkage types that might have shown optical activity, the β - β may be the most likely. The lignans from *Pinus taeda* are reported to be optically active. There is the possibility that even carefully pre-extracted wood meal contains lignans or polymerized lignans. The lack of any optical activity in the β - β -dimer, which was obtained from whole wood, not

isolated lignin, appears to provide evidence in addition to that already at hand that the dimers arise from true racemic lignin, rather than some poly-lignan (that would likely be optically active). Similar conclusions arise from examining CD-spectra of isolated lignins (see below).

Isolated lignins. A valuable and exploitable feature of CD spectra is that CD is associated only with UV bands. Unlike other optical measurements (direct optical rotation, or optical rotatory dispersion), CD spectra can therefore be run on impure samples containing chiral impurities providing the UV-spectra of the component of interest has diagnostic absorptions well separated from those of the chiral contaminants. Isolated lignins always contain

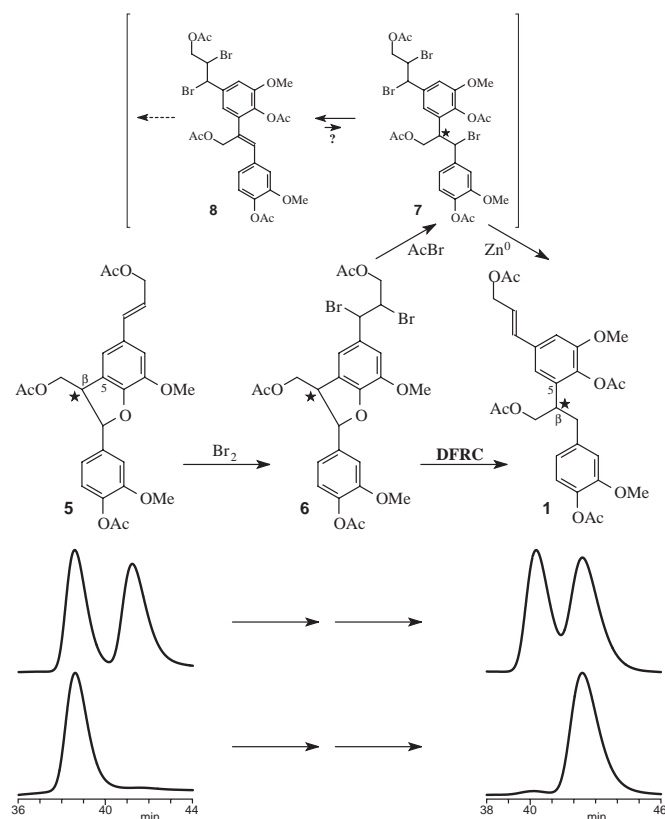


Figure 3. Proof that the DFRC method does not scramble the crucial β -carbon stereochemistry in β -5-units. Dimeric model **5** was first brominated so that the unsaturated sidechain would be generated giving the same β -5-product **1** as isolated from DFRC of lignin. The potential for scrambling the center by HBr elimination from the bromo-intermediate **7** is shown, but as noted in the text, is not anticipated. Chiral HPLC (bottom traces) indicate that a single enantiomer of **5** produces a single enantiomer of **1** following DFRC, proving that the optical center is not scrambled.

contaminating carbohydrates, which are optically active. However, the UV spectra of carbohydrates do not extend much above 200 nm. A major lignin peak of interest is at 280 nm, with shoulders out at around 300+ nm. The range 250-375 nm was sufficiently diagnostic for lignins while devoid of carbohydrate interference. CD spectra of maize, kenaf, pine, and CAD-deficient mutant pine lignins all showed no detectable optical activity, Fig. 3. Improved signal-to-noise was obtained by longer averaging in noisy sub-regions (not shown).

An indication of the level of a chiral component required to observe optical activity can be gained from spiking studies. Fig. 4 shows the CD spectra from pine lignin with ~5% and ~10% levels of the isolated

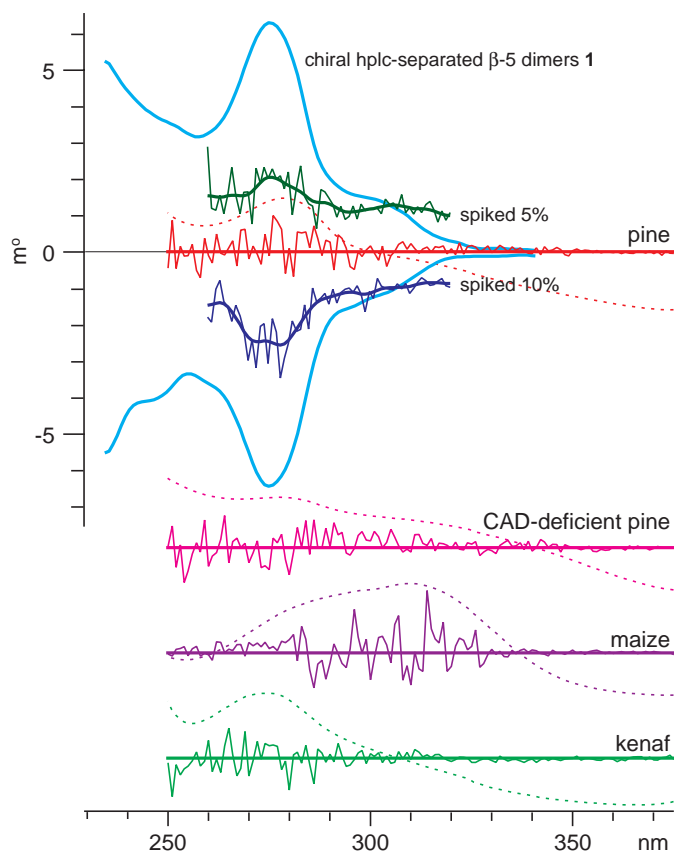


Figure 4. CD spectra of various lignin isolates, illustrating no detectable optical activity. CD spectra of the separated β -5-enantiomers **1** are shown again for comparison. To test the sensitivity of detection, a resolved enantiomer of **1** was spiked into the pine lignin sample at a 5% and a 10% level; the raw data and the curve smoothed by the CD software indicate that a 5% component is reasonably easily detected (although the required level may be higher in the complex lignin sample where various units might have opposing contributions).

chiral β -5 dimer **1** added. The optical activity can be readily seen at the 5% level. Admittedly, with several potential chiral centers in lignin, and the possibility of non-reinforcing optical CD spectra, the ability to determine chirality at levels as low as 5% may still be difficult under our experimental conditions. Unlimited access to long-term-averaging would improve signal-to-noise and should allow an improvement of these determinations.

The maize lignin has high UV absorption centered at ~ 310 nm, resulting in considerable noise in the CD spectrum in that region. The other lignins look rather similar. The CAD-deficient pine mutant was of particular interest because it has been suggested that this isolated lignin is merely a polymerized lignan artifact, an issue that we address more fully elsewhere [see p. 28]. If this were so, the mutant's lignans must be (unexpectedly?) optically inactive since the isolated lignin contains no hint of optical activity.

Conclusions

Our inability to detect optical activity in lignins seems to strengthen the view that lignin is indeed synthesized by the plant without direct (regio- or) stereo-control over the exact course of radical coupling events. An

ability to produce a cell wall polymer with appropriate properties for water transport, defense, and other tasks, with a compositional (and structural) flexibility that is determined by monomer supply is presumably well suited to surviving environmental, gravitropic, and biological stresses. Indeed, the recently noted abilities of plants to remain viable by circumventing biogenetic obstacles placed upon their lignin biosynthetic pathways, in a single generation, without the benefit of evolution, appears to be an endorsement of their flexible strategy. A more extensive discussion of the implications for lignification is in an accompanying article.

References

- Davin, L.B.; Wang, H.-B.; Crowell, A.L.; Bedgar, D.L.; Martin, D.M.; Sarkanen, S.; Lewis, N.G. Stereoselective biomolecular phenoxy radical coupling by an auxiliary (Dirigent) protein without an active center. *Science* 1997, 275, 362-366.
- Eberhardt, T.L.; Bernards, M.A.; He, L.; Davin, L.B.; Wooten, J.B.; Lewis, N.G. Lignification in Cell Suspension Cultures of *Pinus taeda*: In Situ Characterization of a Gymnosperm Lignin. *The Journal of Biological Chemistry* 1993, 268, 21088-21096.
- Lu, F.; Ralph, J. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *J. Agric. Food Chem.* 1997a, 45, 2590-2592.
- Lu, F.; Ralph, J. The DFRC method for lignin analysis. Part 1. A new method for β -aryl ether cleavage: lignin model studies. *J. Agric. Food Chem.* 1997b, 45, 4655-4660.